UPTAKE OF ESTRONE, ESTRADIOL-17ß AND TESTOSTERONE BY ISOLATED RAT LIVER CELLS

Marie Luise Rao, Govind S. Rao and Heinz Breuer
Institut für Klinische Biochemie der Universität Bonn,
5300 Bonn-Venusberg, Federal Republic of Germany

Received May 11,1977

SUMMARY

Estrone, estradiol-17ß and testosterone are taken up by isolated rat liver cells by a process which is saturable. The uptake was linear up to 10 s at 15°C and up to 2 mg cell protein/ml. Under initial uptake conditions a 20 fold accumulation inside the cells was observed. Uptake of all three steroids is decreased by 2,4-dinitrophenol and antimycin A. The uptake of estrone and estradiol-17ß was inhibited by sodium ethylmercurithiosalicylate, iodoacetate, n-ethylmaleimide and 1-fluoro 2,4-dinitrobenzene; uptake of testosterone was not influenced by these reagents. ß-Glucosidase and ß-galactosidase inhibited the uptake of the three steroids by about 30%. Phospholipase A inhibited the uptake of estrone, estradiol-17ß and testosterone 39, 34 and 28%, respectively. The results suggest that steroid hormones enter the cells by pathway(s) in which energy, proteins, sugars and lipids appear to be involved.

One of the prerequisites of steroid hormone action is the crossing of the plasma membrane prior to the expression of its biological activity. However, the mechanisms of translocation of steroids across the membrane, the processes on and in the membrane and inside the cell regulating the influx are still subjects of conjecture. It has been generally assumed that steroids diffuse passively into cells; it has also been speculated that intracellular receptor proteins which are presumed to be bound to the inside of the plasma membrane pick up the hormones from the circulatory system (1). However, work in our laboratory has shown, that intracellular receptors are not directly involved in the uptake of extracellular steroids, but that proteins in the plasma membrane translocate the extracellular steroid into the cell (2). There is increasing evidence indicating that steroid hormones are transported by components in the plasma membrane of liver (3-6), endometrium (5) and uterus cells (7).

In the course of uptake studies of cortisol by isolated rat liver cells (2,3) the question arose whether sex hormones for which the liver can be regarded primarily as a metabolizing organ diffuse into the cell or mechanisms exist involving proteins in the plasma membrane for the transport of these hormones into cells. The pre-

Copyright © 1977 by Academic Press, Inc.
All rights of reproduction in any form reserved.
ISSN 0006-291X

sent studies suggest that uptake of estrone, estradiol-17ß and testosterone by viable rat liver cells is mediated by components in the plasma membrane.

MATERIALS AND METHODS

Adult male rats of the Wistar strain, weighing between 250-300g were used in all experiments. Collagenase, type II was purchased from Boehringer Mannheim GmbH, Germany. [6,7-^{3}H]Estrone (31.4 Ci/mmol), [6,7-^{3}H]estradiol-17ß (41 Ci/mmol), [1,2-^{3}H]testosterone (45.4 Ci/mmol), and inulin-[carboxylic acid-^{14}C] (6.1 mCi/mmol) were purchased from the Radiochemical Centre, Amersham,England.

Isolation of liver cells was carried out as described in a previous communication (3). The sediment of washed cells was suspended in Krebs-Ringer phosphate buffer, pH 7.4. The viability of cells was tested by the exclusion of trypan blue. Cells possessing a viability of 85-95% were used in the present experiments. The amount of liver parenchymal cells was expressed as mg cell protein, estimated by the method of Lowry et al. (8) with crystalline bovine serum albumin as standard; 654,000±16,000 cells corresponded to 1 mg of protein. The gluconeogenic capability and examination of the morphology of cells with the electron microscope has been described before (3).

Measurement of uptake of steroids was carried out as described for the uptake of cortisol (3). Increasing concentrations of non-labelled estrone, estradiol-17ß or testosterone, made by serial dilution, and a constant amount of the respective tritiated steroid ($1 \cdot 10^5$ cpm) in 1.0 ml of Krebs-Ringer phosphate buffer, pH 7.4, were equilibrated for 15 min at 15°C. The reaction was started by adding 0.1 ml of the cell suspension equivalent to about 550 µg cell protein. The incubation was stopped after 7 s by pipetting 1 ml of the incubation mixture with an Eppendorf automatic pipet and filtering through a Whatman GF/C glass fiber filter (3). The filter was washed twice with 5 ml of ice-cold Krebs-Ringer phosphate buffer to remove adsorbed radioactivity. Measurement of radioactivity associated with cells and calculation of the rates of uptake of hormone have been described before (3).

Treatment of cells with 2,4-dinitrophenol (20 mmol/l) and -SH reagents (1 mmol/l, each) was carried out by preincubating portions of cell suspension for 60 min at 2°C.Cells were also treated for 45 min at 15°C with the following reagents: 2-n-nonyl-4-hydroxyquinolin-N-oxide (NHQNO, 100 µmol/l), antimycin A (100 µmol/l) and carboxyatractyloside (100 µmol/l). The cells were then transferred to the incubation tubes containg the same concentration of the reagents in addition to the steroids.

Treatment of cells with enzymes was performed by exposing them for 10 min at 37°C in an atmosphere of O_2 to neuraminidase (10 µg/ml), ß-glucosidase (50 µg/ml), ß-galactosidase (20 µg/ml),pronase (1µg/ml), phospholipase A (20 µg/ml) and phospholipase D (200 µg/ml). These amounts did not induce crucial alterations in the morphology of the cells. Enzyme treatment was terminated by diluting the mixture with 90 fold excess of ice-cold Krebs-Ringer phosphate buffer, pH 7.4, followed by sedimenting the cells by centrifugation for 5 min at 50g. The sediment was taken up in 4 ml of buffer and used for uptake studies. Cells carried through an identical procedure without enzymes served as controls.

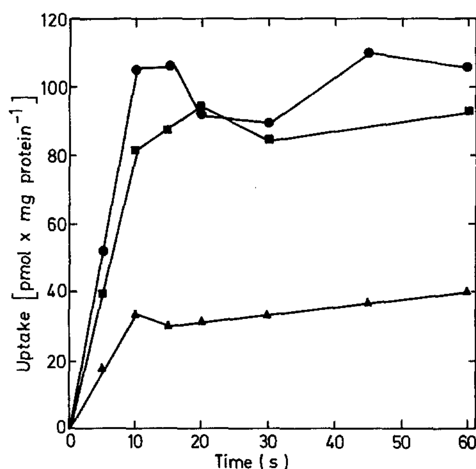

Fig. 1. Time course of uptake of steroids by isolated rat liver cells. Cells equivalent to 1 mg of protein/ml were incubated with 1000 nM steroid at 15°C. At different time intervals 1 ml samples were removed and processed as described in the text. Each value represents the mean of triplicates. The variation coefficients were between 5 and 20%. ■, Estrone; ●, estradiol-17ß; ▲, testosterone.

RESULTS AND DISCUSSION

Dependence of Uptake on the Amount of Protein, Time and Temperature. Uptake of the three steroids (1 µmol/l) by liver cells is linear up to 2 mg of cell protein. At 37°C uptake proceeds very rapidly and accurate measurements of initial uptake rates was not feasible. Therefore the incubation temperature was lowered by stages; at 15°C uptake was linear with time up to 10 s for all three steroids (Fig. 1) and remained constant till 60 min (not shown in the Fig.). All uptake experiments were carried out at 15°C for 5-7 s using 0.5-1 mg of cell protein to ensure working under initial velocity (uptake) conditions. Metabolism of estrone, estradiol-17ß and testosterone under these conditions was not detectable.

Dependence of Uptake on the Concentration of Estrone, Estradiol-17ß and Testosterone in the External Medium. In these experiments total uptake was measured; correction for diffusion and nonspecific binding to cells was not carried out. Steroids in the concentration range of 1-10 nmol/l gave rise to linear rates of uptake; saturability and Michaelis-Menten kinetics were observed in the micromolar range (Fig. 1 A-C). From the reciprocal plot an apparent K_t of 2.2 µmol/l and a V_{max} of 210 pmol/(5 s·mg protein) were calculated for estrone. At lower concentrations of estrone (below 50 nmol/l)

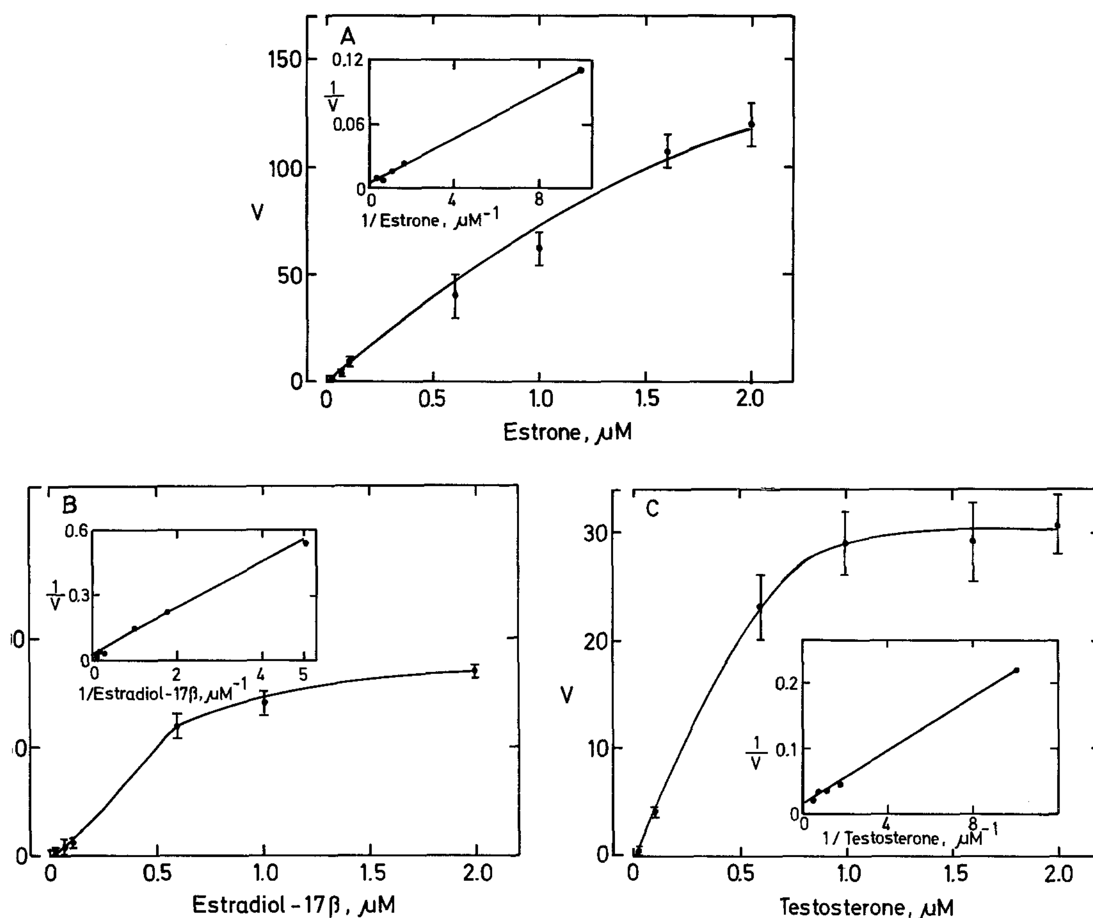

Fig. 2. Dependence of uptake on steroid concentration. Tubes contained increasing concentrations of nonlabelled steroid; A, estrone, B, estradiol-17ß and C testosterone, plus a constant amount of [^{3}H]steroid ($1 \cdot 10^5$ cpm) in Krebs-Ringer phosphate buffer, pH 7.4. Incubation, filtration and washing of cells has been described in the text. Each point in the figure represents the mean of triplicates; vertical bars indicate standard deviation. The insets show double reciprocal plots of the data.

n the medium, the reciprocal plot bends upward, suggesting coope-
atívity (Fig. not shown). Uptake of estradiol-17ß also exhibited
aturation (Fig. 2B); the apparent K_t and V_{max} values were 0.51
mol/l and 46 pmol/(5s·mg protein), respectively. Cooperativity
as obtained with testosterone (below 50 nmol/l) in the external
edium (Fig. 2C); the apparent K_t and V_{max} values from the linear
art of the reciprocal plot (Fig. 2C, inset) were 1.6 μmol/l and

76 pmol/(5s·mg protein), respectively. Since the uptake of the steroids into liver cells proceeded by systems that were saturable, the concentrations of these steroids inside the cells were calculated to find out whether the steroids were transported against a gradient. The intracellular volume was estimated with [4-^{14}C]inulin to be 3-4 µl/mg cell protein. The apparent gradients, inside to outside,were calculated to be 12-23 fold for estrone,15-23 fold for estradiol-17ß and 9-10 fold for testosterone in the concentration range from 20-100 nM of steroid in the external medium.

Influence of Metabolic Inhibitors. Since uptake appeared to proceed against a concentration gradient, information was sought toward involvement of metabolic energy. As can be seen from the data in Table 1, 2,4-dinitrophenol reduces the uptake of estrone and estradiol-17ß to about 20-30% and the uptake of testosterone to about 56-86%. It should be noted that a rather high concentration (20 mmol/l) of the reagent was used in this experiment and effects on the membrane could not be excluded. In order to get more information on the energy requirement of the uptake process other inhibitors (antimycin A, NHQNO, $NaAsO_4$ and carboxyatractyloside) were used. Only antimycin A decreased the uptake of estrone, estradiol-17ß and testosterone to approximately 49-70% (Table All these reagents did not induce any deleterious effects on the plasm membrane. The inhibition by 2,4-dinitrophenol and antimycin A suggests that metabolic energy might partially contribute to the uptake of steroids by liver cells.

Influence of -SH Reagents. In order to test the protein-nature of the carrier involved in the uptake process, liver cells were incubated in the presence and absence of -SH group blocking reagents. Table shows that estrone and estradiol-17ß are inhibited to the same extent by the four different reagents. The influence of these reagents on the uptake suggests that -SH groups are involved in the uptake of estrone and estradiol-17ß and that these systems differ from those involved in the uptake of testosterone,which is not influenced.

Influence of Enzymes Reacting with Plasma Membrane Components. Uptake of a solute from the external medium is a membrane linked process In order to study the involvement of different components on and in th plasma membrane of the rat liver cell in the uptake of sex hormones, treatment of liver cells with various enzymes prior to measurement of uptake was carried out (Table 3).Neuraminidase does not influence the uptake of the sex steroids. Incubation of liver cells with proteolytic

Table 1. Influence of 2,4-dinitrophenol and antimycin A. Preincubation of cells (1 mg protein/ml) with 2,4-dinitrophenol (20 mmol/l) and antimycin A (100 μmol/l), incubation, filtration and counting were carried out as described in the text. The activity in this and the following tables is given as % ± S.D.; the determinations were carried out in triplicate. Uptake in the absence of the inhibitors is taken as 100%.

Steroid	Concentration (nmol/l)	Uptake (%) after treatment with	
		2,4-Dinitrophenol	Antimycin A
Estrone	20	30.5 ± 5.2	64.5 ± 1.5
	100	30.7 ± 3.7	49.4 ± 6.9
	1000	21.8 ± 2.6	69.9 ± 6.8
Estradiol-17ß	20	24.8 ± 3.0	60.4 ± 8.6
	100	26.0 ± 5.7	61.7 ± 5.5
	1000	20.2 ± 1.2	52.5 ± 4.1
Testosterone	20	64.3 ± 8.0	46.2 ±12.8
	100	84.0 ± 9.3⁺	50.9 ± 8.4
	1000	56.0 ± 7.3	65.7 ± 7.2

⁺Difference from control not significant (P>0.05)

Table 2. Influence of -SH reagents. Preincubation of cells (500 μg protein/ml) with -SH reagents (1 mmol/l, each), incubation with steroids (1 nmol/l, each), filtration and counting was carried out as described in the text. Uptake by cells not treated with reagents is taken as 100%.

-SH reagent	Uptake (%)		
	Estrone	Estradiol-17ß	Testosterone
Sodium ethylmercurithiosalicylate	73.2±1.5 (3)	62.1±7.0 (3)	95.1±23.1 (3)⁺
Iodoacetate	78.0±5.0 (3)	79.3±9.3 (3)	94.3±20.1 (3)⁺
1-Fluoro 2,4-dinitrobenzene	57.9±10.4(3)	55.4±16.7(3)	105 ±23.0 (3)
n-Ethylmaleimide	53.5± 4.0(3)	50.4±11.2(3)	94.1±25.9 (3)⁺

⁺Difference from control not significant (P>0.05)

enzymes such as trypsin and pronase resulted in immediate aggregation of cells, when amounts above 10 μg enzyme/ml were used. Low concentrations of pronase (1μg/ml) did not significantly influence the uptake of the hormones. Phospholipase A produces the highest inhibition whereas phospholipase D treatment did not have any significant influence. Harri-

Table 3. Influence of enzymes. Treatment of cells (500 µg protein/ml) with enzymes, incubation with steroids (1 nmol/l), filtration and counting was carried out as described in the text. Uptake by untreated cells is taken as 100%.

Enzyme	Amount	Uptake (%)		
	(µg/ml)	Estrone	Estradiol-17ß	Testosterone
Neuraminidase	10	92.9±3.3 (3)+	83.4±6.9 (3)+	98.9±3.0 (3)+
ß-Glucosidase	50	72.5±1.4 (3)	73.3±2.2 (3)	77.9±7.6 (3)
ß-Galactosidase	20	65.9±4.0 (3)	73.1±5.2 (3)	86.9±2.7 (3)
Pronase	1	91.7±4.2 (3)+	84.0±8.4 (3)+	89.9±4.0 (3)+
Phospholipase A	20	61.1±3.8 (3)	65.7±3.9 (3)	71.6±1.4 (3)
Phospholipase D	200	89.7±2.4 (3)+	86.7±4.7 (3)+	88.9±3.1 (3)+

+Difference from control not significant (P>0.05).

son, Fairfield and Orth (9) have also shown that treatment of AtT-20/D-1 cells with phospholipase A_2 led to a diminished rate of uptake of triamcinolone; treatment with neuraminidase however, did produce an inhibitory effect on the uptake, whereas in the present studies neuraminidase did not show any effect. Hence it appears that glucose and galactose, but not sialic acid residues of the plasma membrane may be involved in the uptake of sex hormones from the external medium; lack of effect of proteolytic enzymes does not indicate noninvolvement of protein components, as they might not be accessible to the enzymes. Perturbation of the lipid portion of the membrane, however, leads to inhibition as visualized by the effect of phospholipase A.

CONCLUSIONS

The wide spread presumption of entrance of steroid hormones into cells of target and nontarget organs by simple diffusion requires to be seriously questioned. Our results show that in addition to diffusion a more interesting entrance pathway exists at the level of the plasma membrane. It remains to be seen if this pathway is an additional link in the chain of mechanism of hormone action.

REFERENCES

1. Jackson, V.& Chalkley, R. (1974) J. Biol. Chem. 249, 1627-1636
2. Rao, G.S., Schulze-Hagen, K., Rao, M.L. & Breuer,H. (1976) J. Steroid Biochem. 7, 1123-1129

3. Rao, M.L., Rao, G.S., Höller, M., Breuer, H., Schattenberg, P.J. & Stein, W.D. (1976) Hoppe-Seyler's Z. Physiol. Chem. 357, 573-584
4. Breuer, H., Rao, M.L. & Rao, G.S. (1974) J. Steroid Biochem. 5, 359
5. Pietras, R.J. & Szego, C. (1977) Nature 265, 69-72
6. Suyenitsu, T. & Terayama, H. (1975) Endocrinology, 96, 1499-1508
7. Milgrom, E., Atger, M. & Baulieu, E.-E. (1973) Biochim. Biophys. Acta 320, 267-283
8. Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) J. Biol. Chem. 193, 265-275
9. Harrison, R.W., Fairfield, S. & Orth, D.N. (1974) Biochem. Biophys. Res. Commun. 61, 1262-1267